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We have finished the construction of the ERα targeting vector and produced ES cell lines. The details of which are provided in the body of this progress report.

The KO construct was electroporated into J-1 ES cells. 120 single clones were obtained after selecting the transfected cells with G418. The ES clone genomic DNA is being screened by PCR with ERKO 3' primers to detect the 5.2kb targeted ER-α allele. The targeted allele will be confirmed with ER/TK 3'-Neo primers, ERKO 5'-Neo primers and Southern blot with the 5' probe. The confirmed positive clone will be treated with Cre recombinase to delete the TK-Neo cassette. Following are the highlights of the progress report.

- Establishment of breast cancer cell lines expressing different ERs.
- Identification of the target genes of estrogen and BPA in breast cancer cell line expressing only ERα.
- Contribution the F-domain of ERα in the estrogen-dependent recruitment of coactivators.
- Construction of the ERα targeting vector and generation of ES cell lines for generating conditional ERα KO mice.

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INTRODUCTION

Breast cancer is among the most devastating diseases affecting women. The risk for a North American women getting breast cancer has doubled since 1940, and at present one woman in eight is at risk of developing the disease. It is generally believed that estrogens play a significant role in the development of breast cancer. The mitogenic action of estrogen is amplified by the hormone-dependent transcription factor, estrogen receptor (ER). Because of its pivotal role in the normal physiology of breast, ER has become a target for pharmacological intervention in breast cancer. This contention is strengthened by observations that the estrogen receptor is a hormone dependent transcription factor that regulates the expression of growth factors and protooncogenes in breast tumor cell lines. Moreover, the growth and progression of many breast cancers are dependent upon estrogen, making measurement of ER-α standard in the treatment decisions for patients with breast cancer. The ER-positive breast tumors are generally associated with faster growing and more aggressive tumors than the ER-positive tumors, which can be controlled with antiestrogen therapy. Towards understanding the role of ER in breast tumorigenesis, we have initiated a program, using transgenic mouse technology to ablate ER-α gene in the mammary gland. This mouse model is expected to be of great use in addressing the role of estrogen receptor in mammary tumorigenesis.

In the previous Progress Report we described our work with ERα mutant (K303R); yeast two-hybrid studies with ERα /ERβ heterodimerization and non-genomic action of estradiol. In the present report, we will only present the progress summary. The current Progress Report will focus on our accomplishments related to the main focus of the DOD-funded project, i.e. generation of conditional ERα knock out (KO) mice.

BODY

1. Study on human ER-K303R mutant:
We have used the cDNA encoding ER (K303R) to stably transfect an ER null cell line called C4-12, using the G418 selection system. The stable cell line was tested for expression of the transgene by Western blot analysis.

2 Protein-protein interactions between ERα and ERβ.
We have expanded the yeast two-hybrid experiments to study the interaction between ERs and the receptor coactivators and concluded that the F-domain of the receptor affects the estrogen-dependent interactions between p160 coactivators and the estrogen – bound ERs.

3. Studies with C4-12 breast cancer cells.
In this study, our most exciting achievement has been the establishment of breast cancer cell lines expressing ERα, ERβ, K303R or ERα/β. We have now systematically begun to analyze these cell lines to determine the target genes for ERs. In the first set of such an experiments, we challenged the ERα expressing cell line with vehicle, estradiol or an environmental estrogen bis phenol-A (BPA)

and analyzed the gene expression pattern with microarray technology. As presented in Table-1, an interesting expression pattern of the target genes emerged. The intriguing observation is that some genes that are induced by estradiol are actually inhibited by BPA and the two ligands showed a different expression patterns. We have further confirmed the expression of some of these genes by real time PCR and focused on one of the protooncogenes, c-myb whose expression has been previously seen in breast cancer patients. We have cloned and sequenced its promoter, which revealed no consensus estrogen response element (ERE). It is likely that c-myb is induced by estradiol and BPA through AP-1 promoter elements.

5. Estrogen receptor-α gene-targeting vector and ES cell lines:
We have finished the construction of the ERα targeting vector and produced ES cell lines. The details are provided below:

Estrogen Receptor-α gene targeting vector: Construction & characterization.

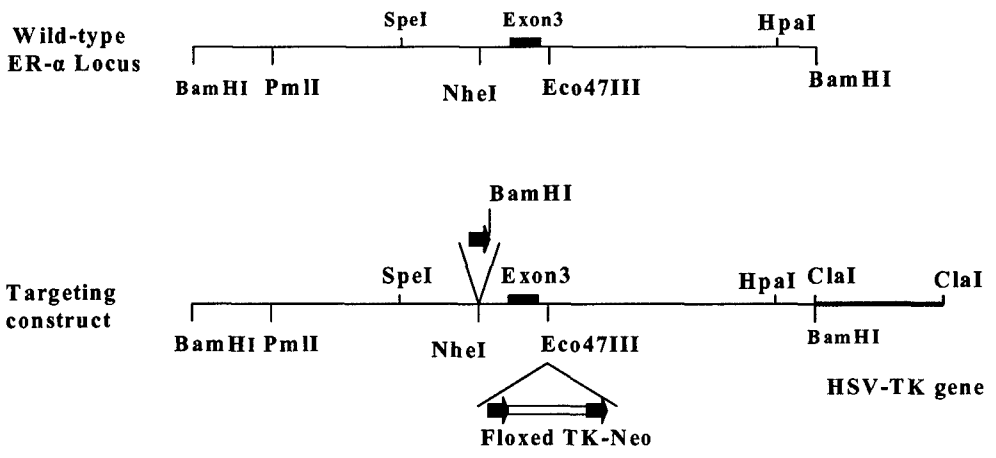

Figure 1 Wild-type ER-α exon3 locus and targeting construct

In order to make the targeting vector, we originally focused to delete exon 2 and obtained a mouse genomic DNA clone harboring a 10kb BamHI fragment of mouse ERα. We however revisited the issue and decided to focus on deleting exon 3. This was prompted by the fact that knockout mice carrying exon 2 deletion in the genomic KO (reported by Ken Korach) some low levels of truncated ERα expression persisted.

We obtained the exon 3 DNA fragment (pBC-ERα-BamHI) from Jan Ake Gustafsson (Karolinska Institute). The pBC-ERα-BamHI construct was amplified

and analyzed by restriction enzyme digestion and sequencing (fig. 1). We have confirmed that the 9.2 kb insert is the ERα genomic fragment and harbors exon3 (fig. 2).

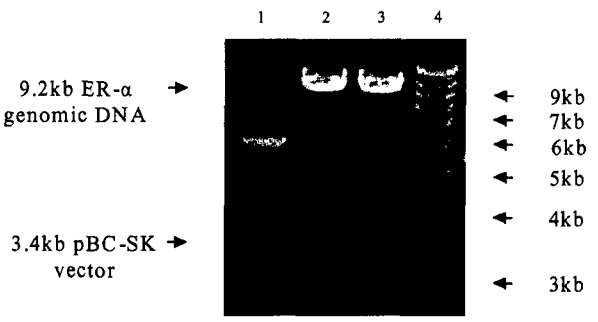

Figure 2 pBC-Erα 9.2kb plasmid digestion. Lane 1: BamHI and SpeI digestion. Lane 2: BamHI digestion. Lane 3: BamHI and NotI digestion. Lane 4 1kb marker (Promega).

Utilizing a series of cloning steps, we designed the ERα conditional Knockout-targeting construct according to a scheme presented in Figure1. In order to screen the positive ES cell clones more efficiently, we introduced a negative selection gene, HSV TK, into the Cla-I site of pBCSK vector (Stratagene). The "Floxed TK-Neo cassette" was inserted into the Eco43III site on the 3' side of exon3. The 46bp LoxP-BamHI fragment was inserted into the NheI site on the 5' side of exon3. The targeting construct was analyzed for its accuracy by restriction enzyme analysis and DNA sequencing (fig 3). The complete sequence of the 12.6 kb "targeting construct' was sequenced from 5' and 3' ends of the genomic DNA insert. We then compared this DNA sequence with the Celera gene bank using the BLAST software and found 97% sequence homology with the mouse genome. At this stage we decided to electroporate the targeting construct into the ES cells

<u>Electroporation of the targeting construct into ES cells.</u>

The KO construct was first linearized with PmlI and electroporated into J-1 ES cells. 120 single clones were obtained after selecting the transfected cells with G418 at a concentration of 240μg/ml. The ES clones were cultured in 24-well plate to full confluency and lysed overnight for the isolation of their genomic DNA. PCR primers were designed to detect the entire targeting construct (fig. 4). The ES clone genomic DNA is being screened by PCR with ERKO 3' primers to detect the 5.2kb targeted allele. The targeted allele will be confirmed with ER/TK 3'-Neo primers, ERKO 5'-Neo primers and Southern blot with the 5' probe. The

confirmed positive clone will be treated with Cre recombinase to delete the TK-Neo cassette.

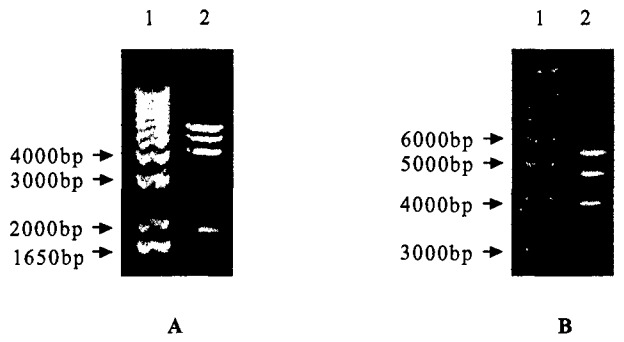

Figure 3 pBC-ERαKO construct BamHI digestion. Two BamHI sites have been introduced into the KO construct at LoxP-BamHI site and the 3' end of Floxed TK-Neo cassette. 4 bands are generated by BamHI (1.9kb, 3.9kb, 4.7kb and 5.4kb). Panel B is a longer run of Panel A. Lane 1 is Promega 1kb marker. Lane 2 shows KO construct BamHI digestion.

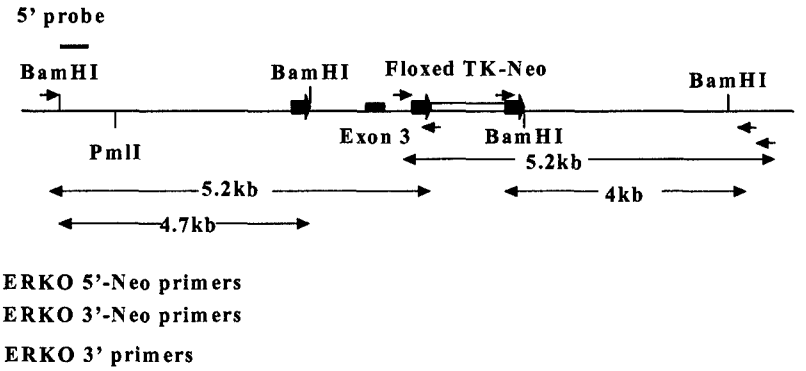

Figure 4 Strategies to screen and confirm the targeted allele. The ES clones will be Screened with ERKO 3' primers for 5.2kb targeted allele. The targeted allele will be confirmed with ERKO 3'-Neo primers, ERKO 5'-Neo primers and Southern blot with 5' probe.

Proposed Changes for the Future Work:

We are not proposing any changes in the experimental plan. After confirming the integration of the targeting construct into the ES cells, they will be used to generate the mice carrying Floxed ERα gene. Once the colony is established, they will be crossed with transgenic mice carrying Cre recombinase gene under the why acidic protein (WAP) promoter to obtain ERα conditional KO mice.

Key Research Accomplishments:

- Establishment of breast cancer cell lines expressing different ERs.
- Identification of the target genes of estrogen and BPA in breast cancer cell line expressing only ERα.
- Contribution the F-domain of ERα in the estrogen-dependent recruitment of coactivators.
- Construction of the ERα targeting vector and generation of ES cell lines for generating conditional ERα KO mice.

REPORTABLE OUTCOMES

Singleton, D., and Khan, S. "Estrogen receptor dependence of MAP-kinase activation in breast and uterine tumor cells Annual Meeting of the "ENDOCRINE SOCIETY", Denver, Co (2001)

Singleton DW, Feng Y, Burd C, Khan SA. Activation of Ras/ERK1/2 by Estrogen Receptor Ligands is Necessary But Not Sufficient to Promote DNA Synthesis in a Human Endometrial cell line. (Manuscript submitted to Endocrinology)

Singleton DW and Khan SA. Xenoestrogen Exposure and Mechanisms of Endocrine Disruption. (Manuscript submitted to Frontiers in Bioscience

Singleton DW, Feng Y, Burd C, Khan SA. Nongenomic Action of Estrogen Receptor Ligands Is not Sufficient to Induce Proliferation of Human Endometrial Adenocarcinoma Cells. (Abstract submitted for presentation at The Endocrine Society Meeting, June 2002)

CONCLUSIONS

Estrogen receptor plays a key role in breast cancer. However, little is known about the involvement of the receptor in the progression of breast tumorigenesis. Towards achieving our overall goal of generating ER knockout mice for studying mammary tumorigenesis, we had proposed to generate ER-α conditional knockout mice. We have successfully generated the targeting construct for use in generating ERα knockout mice. We have also worked on generating ER-specific breast cancer cell lines towards understanding the role of different ER dimmers in estrogen signaling. Using microarray analysis we have identified specific genes for ERα. Finally, we have used the yeast genetic system to explore the role of the F-domain of ER in the recruitment of coactivators for the control of estrogen gene expression.